Summing up, we present a previously unreported mutation in exon 18 of the *c-KIT* gene, contributing to a phenotype of CM with sMCAS and a tendency to incomplete resolution in adulthood. We also identified an additional mutation in exon 18 of the *c-KIT* gene possibly associated with a more severe phenotype.

The study has been approved by the institutional ethics committee (Ethics committee of the Medical University of Vienna, approval number 901/2009). Informed written consent was given by all patients and their parents, respectively. The study adhered to the Declaration of Helsinki principles.

DNA from formalin-fixed paraffin-embedded (FFPE) skin biopsies were extracted using the DNAeasy Blood and Tissue Kit (Qiagen, Valencia, CA) after deparaffinization. For DNA extraction from blood, 200 µl of blood was used with the Qiagen DNA Blood and Tissue Kit according to the manufacturer's instructions. To examine c-Kit exons 8, 9, 11, 13, 17, and 18 for mutations in CM samples, FFPE-derived DNA was further amplified using nested PCR with a multiplex preamplification step to compensate for limited DNA yields from FFPE extraction. Preamplification was performed in a 25-µl volume with the Ampli Taq Gold 360 DNA Polymerase Kit (Applied Biosystems, Vienna, Austria) and at least 25 ng of DNA template. As a second step, amplification using nested primer pairs (see online repository for table) was performed with the Ampli Taq Gold 360 DNA Polymerase Kit and 1 µl of the preamplification product. For amplification steps for blood-derived DNA, the same protocol and primers were used as for FFPE material. After PCR amplification, appropriate exon lengths were controlled on a 1.5% agarose gel, and PCR products were subsequently sequenced using an ABI 3000 capillary sequencer (Applied Biosystems).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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A Method for Intravital Monitoring of Human Cells Using a Far-Red Luminescent Probe in Graft-Versus-Host Disease Model Mice

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TO THE EDITOR

In the past few years, intravital imaging has been developing, especially that using a multiphoton confocal technique (Li *et al.*, 2012). Such a technique is quite a useful tool for understanding cellular or molecular dynamic events *in vivo*. However, this technique requires labeling of fluorescence, and the fluorescence is usually labeled genetically, by transfection, or by direct linking. In the case of human cells, there is the concern that these treatments affect the functional capability of the treated cells. A method of intravital imaging that does not have biological influences is needed.

Recently, we developed a far-red luminescent probe as a convenient

analytical tool for evaluating mAb localization in a living body (Wu *et al.*, 2009). We conjugated a far-red fluorescent indocyanine derivative to biotinylated *Cypridina* luciferase. This conjugate, which is linked to a mAb against a specific antigen, enabled us to obtain high-resolution microscopic images of live, specific antigen-expressing cells without an external light source, and to monitor the accumulation of the probe in mice.

In this paper, we used this probe to detect human cells intravitally in human skin lesions of graft-versus-host disease (GVHD) model mice.

This far-red luminescent probe for human CD45 (anti-human CD45 Ab; BD Biosciences, San Diego, CA) and CD4 (anti-human CD4; BioLegend, Tokyo, Japan), was generated as we reported previously (Wu *et al.*, 2009). Full-thickness human skin from a volunteer was grafted to the backs of immunocompromised NOD/Shi-*scid*, IL-2R^{null} (NOG) mice that lacked T, B, and natural killer cells, allowing a tolerance to human cells (Ito *et al.*, 2002). After confirming engraftment 14 days after skin graft, 1×10^7 human peripheral blood mononuclear cells (PBMCs) from other volunteers were injected intravenously (i.v.) into the skin-grafted mice. For *in vivo* imaging, far-red luminescent probes were injected into the mice i.v. 5 days after human PBMC injection. To obtain the bioluminescence images, the mice were given i.v. injections of *Cypridina* luciferin 24 h before imaging. Bioluminescence imaging was performed by a Photon Imager (Biospace Lab, Paris, France) equipped with an intensified charge-coupled device camera after luciferin injection with 30-s exposure (detailed materials and methods are provided in the Supplementary Data online).

First, we observed human PBMCs incubated with these probes *in vitro* using a charge-coupled device imaging system in a 96-well plate. We determined a light intensity threshold to indicate a positive result (Figure 1a). Next, we injected human PBMCs that had been incubated with these probes *in vitro*. Two hours after injection, concanavalin A (ConA) (20 or 2 mg kg^{-1}) was applied intradermally in the ears of the same mice to induce an inflammatory response. As shown in Figure 1b, higher bioluminescence intensity was detected in the 20 mg kg^{-1} ears (arrowhead) than in the 2 mg kg^{-1} ears (arrow). In addition, the density of infiltrated human CD45$^+$ cells in the 20 mg kg^{-1} ears (Figure 1d) was significantly higher than that in the 2 mg kg^{-1} ears (Figure 1c). These data clearly indicate that the accumulation of labeled cells tends to correlate with bioluminescence intensity. Although the bioluminescence signal disappeared within 30 min, another luciferin application elicited the signal even 2 days later (Supplementary Figure S1 online). These data show that the far-red bioluminescent probe is useful for intravital monitoring of human cells in mice.

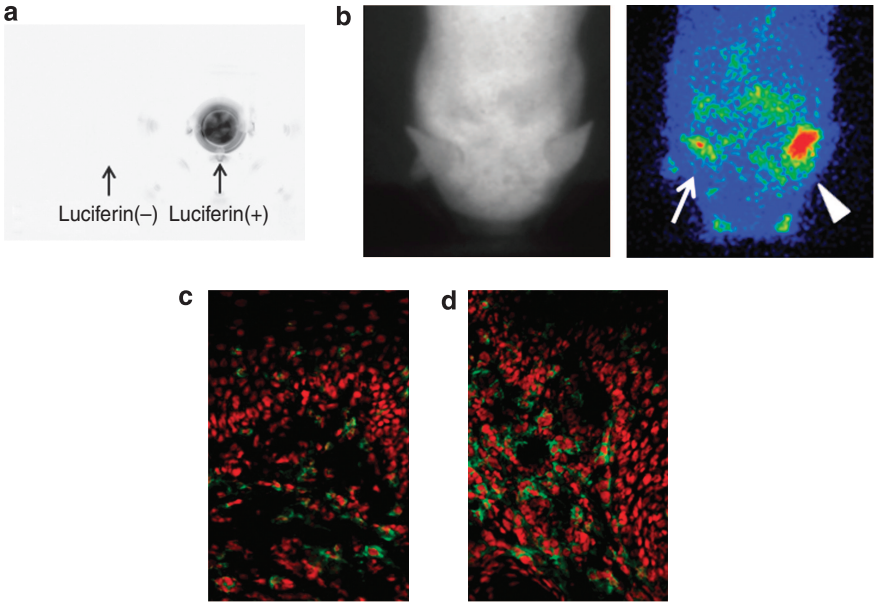

Figure 1. Bioluminescence images of a far-red luminescent probe *in vitro* and *in vivo*. (**a**) Human peripheral blood mononuclear cells (PBMCs) incubated with the probes *in vitro* were observed using a charge-coupled device imaging system in a 96-well plate. The detected light intensity of the probe with luciferin was high. (**b**) Human PBMCs that were incubated with these probes *in vitro* were injected into NOG mice. Two hours after injection, 2 mg kg^{-1} (arrow) or 20 mg kg^{-1} (arrowhead) of ConA was injected intradermally in the ears of the same mouse. Next, luciferin (200 µl) was injected after 1 day. Bioluminescence images showed the probes to be visible in the ConA-injected ear, suggesting that human CD45$^+$ PBMCs had accumulated there. Immunostaining showed numerous infiltrating human CD45$^+$ cells in treated ears, and the density of infiltrated human CD45$^+$ cells in the 20 mg kg^{-1} ear (**d**) was significantly higher than in the 2 mg kg^{-1} ear (**c**).

Next, to evaluate the usefulness of this probe in detecting human cell behavior in mice, we examined the distribution of human PBMCs that were injected into human skin lesions of GVHD model mice (Figure 2a) using this probe with anti-human CD45 Ab. Bioluminescence images showing a high signal were specifically detected in the skin-grafted areas (Figure 2b). The NOG mice in our experiment are severely immunocompromised, and lymph nodes could hardly be identified (Kambe *et al.*, 2004). Therefore, human lymphocyte accumulation was observed only in the human skin–grafted area. Six days after human leukocyte injection, there were no manifestations and no human cells were detected in other skin using human CD45 staining (data not shown). Immunostaining confirmed that numerous infiltrating human CD45$^+$ cells were accumulated in the skin graft (Figure 2c). A few murine dendritic cells (DCs) (murine CD11c+) were detected in the upper dermis of the GVHD area, whereas numerous human T cells (human CD3+) were infiltrated in the same area (Supplementary Figure S2 online). In contrast, there was a certain number of human DCs (human CD11c+) in the GVHD area, and some human DCs seemed to locate closely to human T cells (Supplementary Figure S3

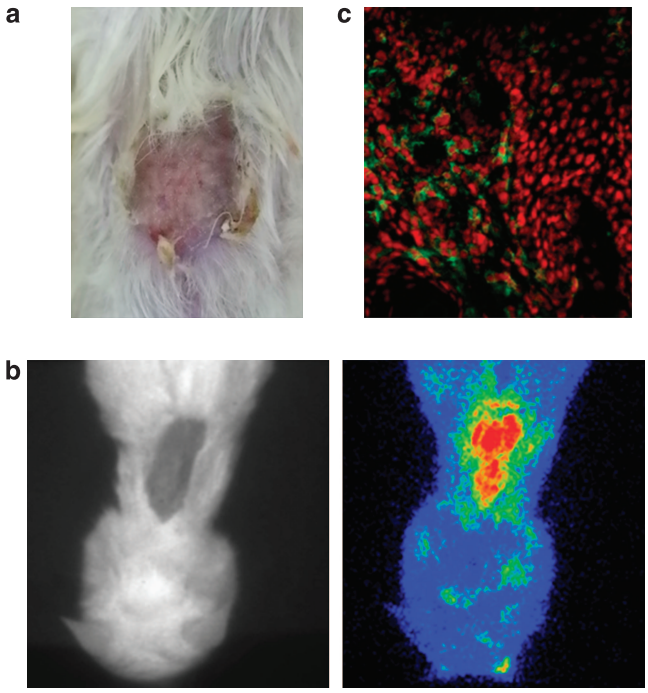

Figure 2. Visualization of human cells in graft-versus-host disease (GVHD) lesion using a far-red luminescent probe. (**a**) The human skin graft on a NOG mouse. (**b**) Ten million peripheral blood mononuclear cells in 200 μl of phosphate-buffered saline per mouse was injected i.v. For *in vivo* imaging, a 0.2-ml solution containing anti-CD45 antibodies was injected into the mice i.v. 24 h before imaging. Each experiment was carried out twice. To obtain the bioluminescence image, the mice were given injections of 200-μl *Cypridina* luciferin just before imaging. Bioluminescence imaging was performed by Photon Imager equipped with an intensified charge-coupled device camera after luciferin injection into the GVHD model mice. Bioluminescence images were specifically detected in skin-grafted areas. (**c**) Skin lesion of GVHD model mice stained with FITC anti-human CD45 Ab. The immunostaining confirms that numerous infiltrating human $CD45^+$ cells have accumulated in the skin graft.

online). From these data, it is possible that human T cells and human DCs interact in the skin lesion of this GVHD model. Probes with anti-human CD4 or CD8 were also able to demonstrate the gathering of cells in the skin-grafted areas, confirming the infiltration of both cell types into the skin-grafted areas (Supplementary Figures S4 and S5 online). These data indicate that the far-red bioluminescent protein probes enable the monitoring of human cells temporarily in human GVHD skin lesions on model mice.

Fluorescence labeling is an alternative method for detecting human cells; however, labeling treatment might influence biological function. Indeed, modification of a specific gene often affects the expression of other genes or proteins. In antigen–antibody-based fluorescence labeling, signal induction or inhibitory effect by antigen-antibody binding are concerns. Therefore, the choice of antibody is crucial in generating probes. The antibodies that were used in our study have been confirmed to have little effect on cellular function. Furthermore, our probe enables serial intravital observation. Because of the similarity of luciferin to albumin, it does not cause an immune response and the toxicity appears low. However, its usefulness in antibody-based labeling is limited, because it is difficult to separate multiple bioluminescence signals, whereas fluorescence provides several separate signals. In addition, using multiphoton intravital imaging, it is now possible to perform dynamic, multidimensional imaging to track cell populations at the single-cell level *in vivo* (Li *et al.*, 2012). For example, dynamic processes in skin transplant rejection in mice were visualized by multiphoton intravital imaging (Celli *et al.*, 2011). For bioluminescence signals, it is difficult to detect cellular-level imaging at present. If these limitations are overcome, bioluminescence imaging promises to be a powerful tool for revealing biological dynamics.

Furthermore, our data showed that human T cells do not necessarily require lymphoid organs to expand in the murine host, and that they expand in skin. In addition, human T cells associate predominantly with human, rather than murine, DCs, suggesting that DCs of recipient origin contribute to the expansion of T cells in this GVHD model.

In conclusion, our far-red bioluminescent probe promises to enable the analysis of human cell localization in model mice without modification of the cellular function.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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